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Dark-mediated dormancy release in stratified *Lolium rigidum* seeds is associated with higher activities of cell wall-modifying enzymes and an apparent increase in gibberellin sensitivity

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ABSTRACT

Dormancy release in freshly matured, imbibed annual ryegrass (*Lolium rigidum*) seeds is inhibited by light and involves a decrease in seed sensitivity to abscisic acid. Other processes involved in dormancy release in the dark were investigated by measuring seed storage compound mobilisation and the activity of cell wall-degrading enzymes. Activities of endo-β-mannanase and total peroxidase were higher in dark-stratified compared to light-stratified seeds, indicating that weakening of the structures constraining the embryo was accelerated in the dark. A dramatic degradation of storage proteins in light-stratified seeds, accompanied by induction of a high molecular mass protease, suggests that maintenance of storage(-like) proteins is also important in dark-mediated dormancy release. α-Amylase activity was induced in dark-stratified seeds at least 48 h prior to radicle emergence upon transfer to conditions permitting germination, or in light-stratified seeds supplied with exogenous gibberellin A_4. This suggests that (a) α-amylase is involved in stimulation of germination of non-dormant *L. rigidum* seeds, and (b) dark-stratified seeds have an increased sensitivity to gibberellins which permits the rapid induction of α-amylase activity upon exposure to germination conditions. Overall, it appears that a number of processes, although possibly minor in themselves, occur in concert during dark-stratification to contribute to dormancy release.

Introduction

Annual ryegrass (*Lolium rigidum*) seeds, usually dormant at maturity, can lose dormancy either over a period of several months through dry storage (after-ripening) or within a few weeks during fully hydrated stratification in the dark (Steadman, 2004; Steadman et al., 2004). Dark-stratified seeds then initiate germination in response to alternating temperatures and exposure to light for at least 8 h, but light-stratified seeds remain dormant. Dark-mediated dormancy release in imbibed seeds appears to involve a decrease in their sensitivity to abscisic acid once they are placed under conditions promoting germination (Goggin et al., 2009). The role of gibberellins (GA) in ryegrass seed dormancy release/germination remains obscure due to the low levels of endogenous embryo GA and the only very subtle effects of exogenous GA_4 on germination (Goggin et al., 2009). Apart from this information about the interaction of the seeds with plant growth regulators, little is known about the subsequent biochemical changes taking place in *L. rigidum* seeds during dark-mediated dormancy release.

Completion of seed germination requires that the growth potential of the embryo exceeds the mechanical strength of its surrounding tissues (Bewley, 1997a). Therefore, it was hypothesised that reserve mobilisation, to provide energy for embryo expansion, and cell wall hydrolysis, to weaken the structures constraining the embryo, may be activated in *L. rigidum* seeds stratified in the dark, thus contributing to dormancy release. In grasses and crop cereals, the major seed storage compound is starch, digested (mainly) by amylases in a series of hydrolytic steps. α-Amylase, as the first and most critical enzyme of starch breakdown in these seeds, acts upon native starch granules. Although its activity is usually not present in ungerminated crop cereal seeds (e.g. Perata et al., 1992), the fact that *L. rigidum* populations repeatedly selected for low dormancy showed constitutive α-amylase activity (Goggin et al., 2010) prompted an analysis of this enzyme in dark- and light-stratified seeds in the current study.

Abbreviations: DTT, dithiothreitol; GA, gibberellin; Hepes, N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid; ICL, isocitrate lyase; PMSF, phenylmethylsulphonyl fluoride.

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Storage proteins occur in the embryo, aleurone and starchy endosperm of cereal seeds and comprise approximately 5% of the dry mass of a mature seed (Shewry and Halford, 2002). Upon imbibition or just prior to seed germination, storage proteins are mobilised to provide amino acids for the synthesis of new proteins or as a source of nitrogen and carbon for energy-generating metabolic pathways. Although lipids play only a minor role as storage compounds in grass seeds, it has been proposed that oil reserves in the embryo and aleurone of cereal seeds may be utilised first in order to provide energy and substrates for the synthesis of hydrolases and membrane translocation machinery (Eastmond and Jones, 2005). Conversion of mobilised fatty acids to carbohydrates for energy production occurs via the glyoxylate cycle and the first enzyme of this pathway, isocitrate lyase (ICL), is more highly expressed in non-dormant *Arabidopsis* seeds than in their dormant counterparts (Chibani et al., 2006). This suggests that ICL may play a role in mediating seed dormancy.

The other side of the dormancy/germination equation is the weakening of the structures constraining the embryo, such as the endosperm in *Arabidopsis* and other dicotyledonous plants, and the coleorhiza in cereals (Barrero et al., 2009). Endo-β-mannanase is an important enzyme in cell wall hydrolysis, but its role in dormancy/germination appears to vary between species: it is active in the endosperm of *Datura ferox* seeds prior to radicle emergence (Sánchez and de Miguel, 1997), reaches its peak activity in rice seeds only after radicle emergence (Wang et al., 2005), is apparently only involved in substrate mobilisation in lettuce seeds (Bewley, 1997b), and in tomato, differentially localised isoforms play separate roles in seed germination vs. substrate mobilisation (Toorop et al., 1996). The cell wall loosening required for radicle growth and emergence can also be effected by hydroxyl and superoxide radicals, which have been shown to be generated in the apoplast of germinating cress (Müller et al., 2009) seeds by apoplastic peroxidase activity. Peroxidases play a multitude of roles in plants, including cell wall remodelling during elongation and growth, and the evidence for their involvement in seed germination is growing.

In the current study, the potential roles of storage compound mobilisation and cell wall hydrolysis in dark-mediated dormancy release in *L. rigidum* seeds were investigated by measuring carbohydrate, lipid and protein concentrations and the activities of enzymes involved in their mobilisation in dark- and light-stratified seeds, along with the activities of cell wall-modifying enzymes. The processes of dormancy release vs. germination were distinguished by taking measurements either immediately after the stratification treatment, or 24 h after transferral of stratified seeds to germination conditions. This utilises the fact that (low dormancy) dark-stratified *L. rigidum* seeds cannot initiate germination until they are exposed to alternating light and temperature.

Materials and methods

Seed material

Lolium rigidum Gaud. (annual ryegrass) seeds were collected from plants infesting a wheat field at Wongan Hills (30°53′S, 116°43′E) in October 2006, and stored at −20 °C to minimise loss of dormancy through after-ripening. The basal germination characteristics of these seeds and their responses to dark- and light-stratification and plant growth regulators are described in previous work (Goggin et al., 2009). For biochemical extractions, seeds were stratified on 1% (w/v) agar in 9 cm diameter Petri dishes either in the dark, or under 50 W halogen globes with a fluence rate of 90 μmol m^{-2} s^{-1} over 400–700 nm, at 20 °C for 21 d. To assess germination after 0, 7, 14 or 21 d stratification, seeds were transferred to a growth cabinet with a 12 h photoperiod (combined incandescent and fluorescent light with a fluence rate of 45 μmol m^{-2} s^{-1} over 400–700 nm) and day/night temperatures of 25/15 °C for a further 21 d.

Chemicals

All chemicals and enzymes were obtained from Sigma–Aldrich (Sydney, Australia), unless otherwise stated.

Analysis of metabolites

Measurements of water-soluble carbohydrates and starch were performed on seed samples that had been dark- or light-stratified for 0 (i.e. dry seeds), 7, 14 or 21 d; all other measurements were performed on seeds stratified for either 0 or 21 d.

Water-soluble carbohydrates and starch

Seeds were homogenised in liquid nitrogen and then immediately extracted twice in 5 vol water at 90 °C for 20 min. Reducing sugars were measured colorimetrically by their reaction with 3,5-dinitrosalicylic acid (Guglielminetti et al., 1995). Glucose at 0–9 μmol was used as a standard, and recovery of glucose added to extractions was 108 ± 2%. Starch was extracted from the seed residue using DMSO/HCl and then hydrolysed with amyloglucosidase, according to the method of Beutler (1984). The resulting glucose was measured spectrophotometrically using Sigma Glucose Assay Reagent, with 0–15 mM glucose as a standard. Measurements on the water-soluble carbohydrate fraction and on unhydrolysed starch extracts showed that no starch was extracted by incubation of the seeds in water at 90 °C, and no free glucose remained in the seeds following water extraction. The recovery of soluble potato starch added to extractions was 82 ± 3% (data was not adjusted).

Total lipids

Total lipids were extracted using the Folch method as described in Iverson et al. (2001). Seeds were homogenised in 7.5 vol of chloroform:methanol (2:1), and then 0.88% NaCl in water was added to a final ratio of 8:4:3 chloroform:methanol:water. Following centrifugation, the lower phase was transferred to a fresh tube, the solvent removed by evaporation, and the mass of the remaining lipids was recorded.

Proteins

A modified Osborne fractionation (Klose et al., 2009) was used to extract four separate fractions of proteins (water-soluble albumins, salt-soluble globulins, ethanol-soluble prolamins and urea-soluble glutenins) from acetone-defatted seeds. The fractions were concentrated and desalted by acetone precipitation and then resuspended in 2.5 vol (based on seed dry mass) 2× SDS-PAGE sample buffer (Laemmli, 1970). Protein concentration was measured using the method of Bradford (1976) with BioRad Dye Reagent Concentrate, and with 0–25 μg BSA as a standard. Proteins were separated on SDS-PAGE (12% (w/v) polyacrylamide) mini-gels which were stained with colloidal Coomassie Brilliant Blue G-250 (Candiano et al., 2004).

2D-PAGE

All protein extraction steps were performed at 4 °C unless otherwise indicated. An acetone powder was first made from 0.3 g (dry mass) seeds, and soluble proteins were extracted on ice for 2 h in low-salt extraction buffer (20 mM Tris base, 10% (w/v) glycerol, 1% (w/v) Triton X-100, 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonyl fluoride (PMSF). Following centrifugation to remove insoluble material, the soluble proteins were acetone-precipitated at −20 °C. The remaining insoluble proteins were

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extracted in denaturing extraction buffer (8 M urea, 2% (v/v) Triton X-100, 5 mM DTT) at room temperature for 1–2 h, re-centrifuged to remove insoluble material and then methanol-precipitated at −80 °C. Both sets of precipitated proteins were resuspended in isoelectric focusing sample buffer (8 M urea, 2% (w/v) CHAPS, 60 mM DTT, 2% (v/v) IPG buffer, pH 3-10 NL (GE Life Sciences)), and protein was quantified using the modified Lowry method of Peterson (1983), with 0–60 μg BSA as a standard.

Immobiline pH 3-10 NL, 13 cm IPG strips (GE Life Sciences) were rehydrated with 1 mg of sample protein, focused for a total of 17 kVh on a Multiphor II (Pharmacia) and then equilibrated for SDS-PAGE, according to the manufacturer's protocol. Second dimension vertical gels (12.5% (w/v) polyacrylamide, 16 cm × 18 cm × 0.5 cm) were run at 30 mA per gel (4 °C) and then stained in colloidal Coomassie Brilliant Blue G-250 for 72 h.

Enzyme assays

All extractions were performed at 4 °C, and spectrophotometric assays included boiled seed extracts as negative controls. Four replicates of each treatment were measured. For zymogram assays, experiments were performed at least twice, with duplicate gels being run each time. Protein in the seed extracts was quantified using the method of Bradford (1976) as above. Total amylase activity was measured in seed samples that had been dark- or light-stratified for 0, 7, 14 or 21 d; all other activities were measured in seeds stratified for either 0 or 21 d.

Amylolytic activity

Total amylase activity was measured according to Guglielminetti et al. (1995). The reaction at 30 °C proceeded linearly over 20 min, so activity was measured after 15 min. Recovery of *Aspergillus oryzae* α-amylase (EC 3.2.1.1) added to seed extracts was 87 ± 9%. As α-amylase activity was undetectable in *L. rigidum* seeds using this method (data not shown), the more sensitive but semi-quantitative zymogram method was used as described in Goggin and Colmer (2007), except that 30 μg protein was loaded into each gel lane. Control lanes with 0.005 U of α-amylase from *A. oryzae* were run alongside the samples.

Proteolytic activity

Protease activity in seeds was detected using gelatine SDS-PAGE at pH 7 according to Einali and Sadeghipour (2007), with 10 μg of seed protein being loaded per lane. Gels were stained with Coomassie Brilliant Blue R-250 to detect cleared zones in the gelatine. Pilot studies showed that protease activity in all samples was higher at pH 7 than pH 4 (data not shown). Positive and negative control lanes with 0.5 U of proteinase K from *Tritirachium album* or 10 μg BSA, respectively, were run alongside the samples.

Isocitrate lyase

ICL (EC 4.1.3.1) assays were performed at room temperature using a slightly modified protocol from Cooper and Beevers (1969). Reaction mixtures (1 mL) contained 50 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (Hepes), pH 7, 8.7 mM $MgCl_2$, 10 mM phenylhydrazine, 4.6 mM DTT, 50 μL desalted enzyme extract and 13 mM DL-isocitrate, and the change in absorbance at 324 nm upon the addition of isocitrate was monitored for several minutes. An extinction coefficient of 17 mM^{-1} cm^{-1} was used to calculate enzyme activity from the linear range of the reaction.

Cell wall hydrolases

Seeds were homogenised in 3 vol buffer A (100 mM Hepes–NaOH, pH 8), and centrifuged to remove cell debris. The supernatant (soluble fraction) was used to measure endo-β-mannanase (EC 3.2.1.78) activity, with 2 μL being loaded into agarose gels containing 0.1% (w/v) locust bean gum galactomannan according to Bourgault and Bewley (2002). Endo-β-mannanase activity was quantified from the diameter of the galactomannan hydrolysis zone upon staining with Congo Red, using 0–2×10^{-4} units of endo-β-mannanase from *Aspergillus niger* (Megazyme, Melbourne, Australia) as a standard. Tissue-printing for localisation of endo-β-mannanase activity within seeds was performed by dissecting seeds longitudinally, pressing the cut half against a galactomannan plate prepared as above, and incubating at 30 °C for 5–120 min before staining with Congo Red.

The insoluble seed material remaining after extraction with buffer A (above) was extracted in 1.5 vol buffer B (0.1 M sodium citrate, pH 6, 0.1 M KH_2PO_4, 1 M NaCl). The resulting supernatant (salt-soluble, cell wall-bound fraction) was used to measure β-mannosidase (EC 3.2.1.25) activity using the spectrophotometric method of Sánchez and de Miguel (1997), with 0–200 μM *p*-nitrophenol as a standard.

Total peroxidase

Total peroxidase (EC 1.11.1.7) activity in seeds extracted in 100 mM KH_2PO_4 (pH 7.5) containing 1 mM EDTA, 5% (v/v) glycerol, 0.002% (v/v) Triton X-100, 2 mM ascorbate, 5 mM DTT and 1 mM PMSF was measured by following the conversion of guaiacol to tetraguaiacol at 470 nm, according to Chance and Maehly (1955). Boiled seed extracts were used as negative controls. Peroxidase activity was calculated from the change in absorbance in the linear range of the reaction, using an extinction coefficient of 26.6 mM^{-1} cm^{-1}.

Statistical analysis

Differences between quantitative measurements were assessed by one-factor ANOVA and the least significant difference test, at the 5% level of significance.

Results

Seed carbohydrate concentration and amylolytic activity

To give an indication of the progress of dormancy release during dark- or light-stratification, the germination of seeds transferred to alternating light and temperature following 0–21 d stratification is shown in Fig. 1A; by the end of 21 d stratification, the dark-stratified seeds were capable of germinating to 80%, whereas light-stratified seeds only reached 35% germination. Water-soluble carbohydrates extracted from dark- and light-stratified seeds showed a 4-fold decrease between 0 and 7 d after the start of imbibition, and then remained stable until the end of the experiment at 21 d, with no difference between the dark and light treatments (Fig. 1B). Dark-stratification caused an initial 2-fold decrease in seed starch concentration, whilst light-stratification caused no net changes (Fig. 1C). Overall, there was no significant difference between dark- and light-stratified seed starch concentration after the 7 d timepoint.

There was no difference in total amylase activity between dark- and light-stratified seeds over the entire 21 d of the treatment (Fig. 1D). α-Amylase activity was undetectable in dry and 21 d dark- or light-stratified seeds (Fig. 1D inset, lanes 1–3). However, upon transferral of 21 d-stratified seeds to germination conditions for only 24 h (radicle emergence in non-dormant seeds takes 72 h), activity was induced in seeds stratified in the dark but not in the light (Fig. 1D inset, lanes 4 and 5). Application of exogenous GA_4 during the 21 d stratification had no effect in the dark, but induced α-amylase activity in the light (Fig. 1D inset, lanes 6 and 7). Seeds

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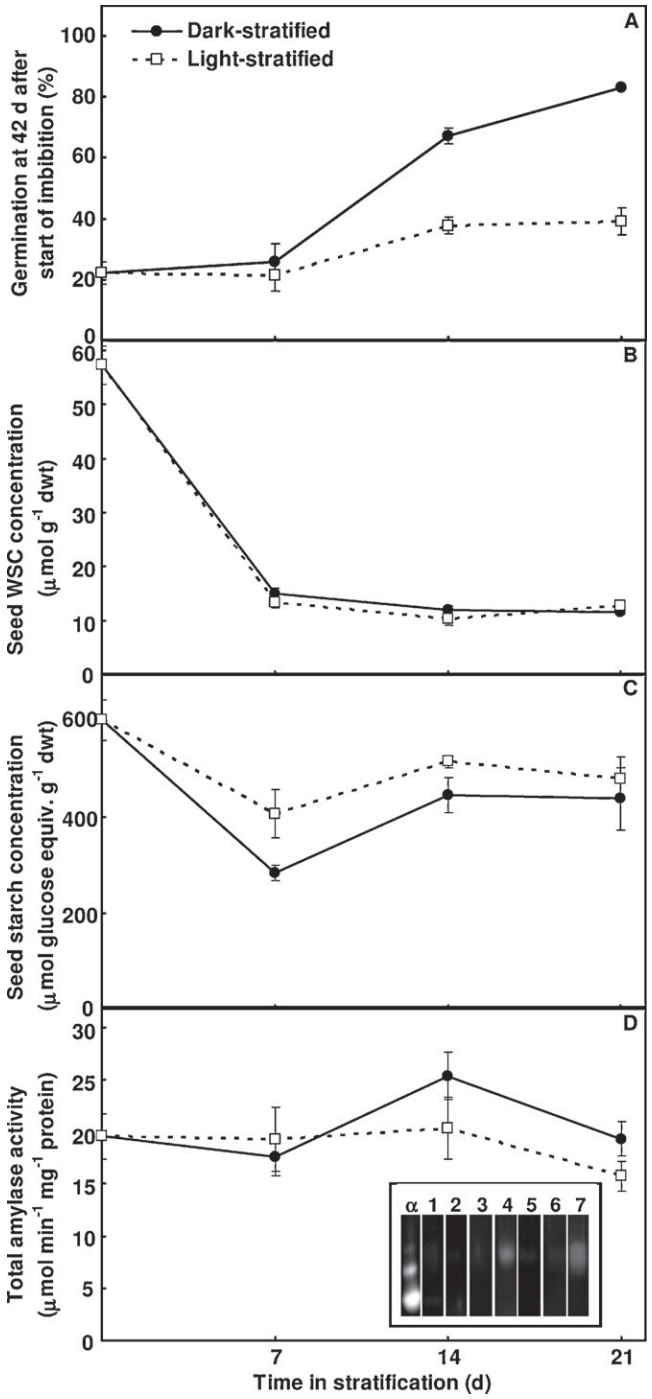

Fig. 1. Changes in germination percentage (A), water-soluble carbohydrate concentration (B), starch concentration (C), total amylase activity (D) and α-amylase activity (D inset) in dark- or light-stratified seeds over 21 d at 20 °C. In (A), germination at 42 d after the start of imbibition was assessed in seeds transferred to germination conditions (25/15 °C, 12 h photoperiod) after 0, 7, 14 or 21 d stratification. In (D inset), the lanes represent: α, commercial α-amylase; 1, dry seeds; 2 and 3, 21 d dark- and light-stratification; 4 and 5, 21 d dark- and light-stratification followed by 24 h in germination conditions; 6 and 7, 21 d dark- and light-stratification with 10 μM GA_4. Values in (A–D) are means ± SE ($n = 4$). Representative gels from two independent experiments, each with two replicates of 50 seeds per sample, are shown in (D inset).

stratified in the light in the presence of GA_4 still required alternating temperature and light conditions for induction of germination (data not shown), in spite of the presence of α-amylase activity prior to germination.

Table 1
Metabolism of lipids, proteins and cell walls during 21 d stratification at 20 °C in the dark (D) or light (L). Concentrations of total lipids and proteins are expressed as mg g^{-1} dwt, and activities of isocitrate lyase (ICL) and β-mannosidase as nmol product formed min^{-1} mg^{-1} protein. Values are means ± SE ($n = 4$); significant differences ($P < 0.05$) between means are denoted by different letters within rows.

	Dry seeds	21 d D	21 d L
Total lipids	18 ± 1[a]	15 ± 2[a]	14 ± 1[a]
ICL activity	24 ± 4[a]	128 ± 21[b]	99 ± 35[b]
Albumins	9.4 ± 1.1[a]	4.1 ± 1.0[b]	3.4 ± 0.4[b]
Globulins	7.0 ± 0.7[a]	8.3 ± 0.9[a]	8.7 ± 0.6[a]
Prolamins	2.7 ± 0.6[b]	1.7 ± 0.5[b]	4.5 ± 0.5[a]
Glutenins	56 ± 4[a]	57 ± 3[a]	65 ± 4[a]
β-Mannosidase activity	12 ± 1[b]	26 ± 0.3[a]	29 ± 1[a]

Seed lipid concentration and ICL activity

The concentration of total lipids in dry seeds decreased slightly, but not significantly, after 21 d stratification, and there was no difference between the light and dark treatments (Table 1). ICL activity increased more than 4-fold upon stratification in both the light and the dark (Table 1).

Seed protein profiles and proteolytic activity

There was little difference between dark- and light-stratified seeds with respect to the concentration of total seed proteins (Table 1), or their SDS-PAGE profiles (data not shown); however, the greater resolving power of 2D-PAGE demonstrated that dark- and light-stratification have markedly different effects on the seed protein profile (Fig. 2 and Supplementary Fig. 1). Light-stratified seeds (Fig. 2C) showed a distinct absence of neutral proteins in the mass range of 45–100 kDa, and extensive degradation of the acidic proteins of 30–50 kDa, that were abundant in the dry (Fig. 2A) and dark-stratified seeds (Fig. 2B). An attempt was made to identify some of the differentially expressed proteins in dark- vs. light-stratified seeds. This was largely unsuccessful due to the low number of genes that have been sequenced in the *Lolium* genome; however, a number of the tentative protein identifications were homologous to seed storage proteins from wheat, oat, rice and *Arabidopsis thaliana* (Supplementary Tables 1 and 2; Supplementary Fig. 1).

Zymograms of seed proteases demonstrated that dry seeds possessed very low levels of protease activity which increased upon stratification (Fig. 2D). The banding pattern of protease activity was different in dark- vs. light-stratified seeds, with the dark-stratified seeds exhibiting a higher number of discrete bands, but the light-stratified seeds showing greater intensity of the bands of >100 kDa (Fig. 2D).

Seed cell wall-degrading activities

The relatively high endo-β-mannanase activity in dry seeds was maintained by 21 d dark-stratification, but decreased by 25% during light-stratification. Seeds transferred to germination conditions for 24 h following stratification showed the same activity as light-stratified seeds, whether they had been stratified in the dark or light (Fig. 3A). Any specific tissue localisation of endo-β-mannanase activity within *L. rigidum* seeds was unable to be determined by tissue-printing on galactomannan plates, as it appeared to be uniformly spread across the whole seed (Fig. 3A, inset). Activity of β-mannosidase, the enzyme following endo-β-mannanase in the degradation of mannans, was also present in dry seeds, and increased equally (>2-fold) upon imbibition in either the dark or light (Table 1).

Total peroxidase activity was present in dry seeds and remained at the same level after 21 d light-stratification; however, activity

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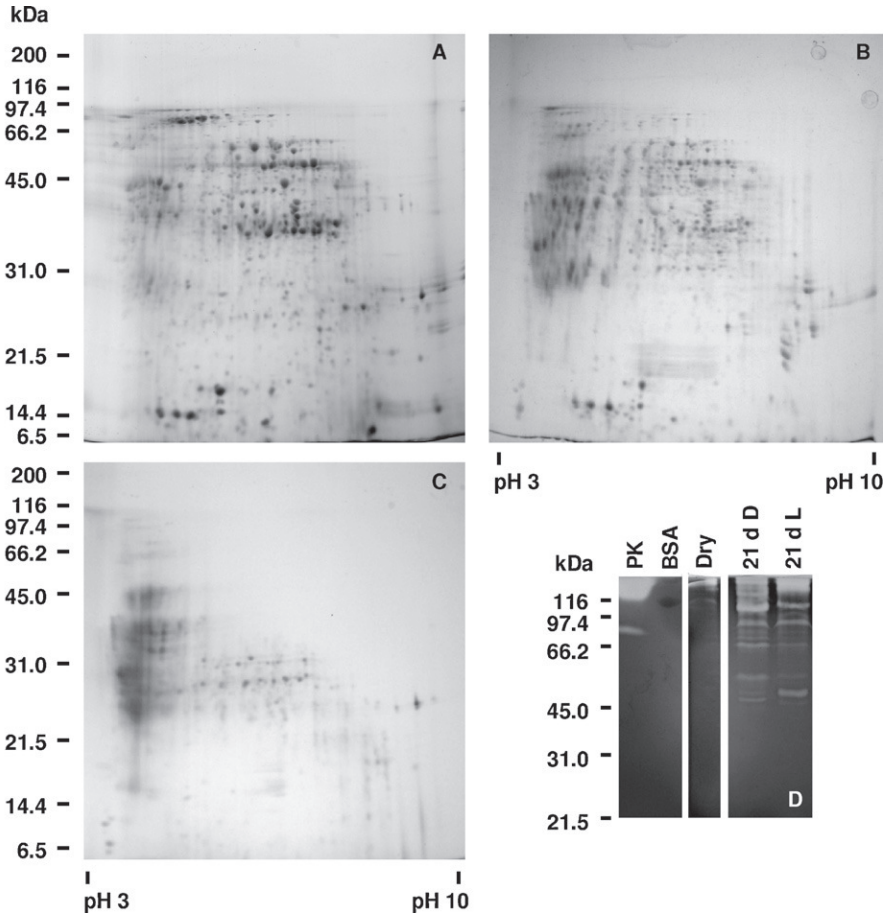

Fig. 2. Soluble protein 2D-PAGE profiles ((A) dry seeds; (B) dark-stratified seeds; (C) light-stratified seeds) and protease activity as detected by gelatine SDS-PAGE (D) in seeds stratified for 0 or 21 d at 20 °C. Four biological replicates for each treatment were run on separate gels; representative gels are shown. Molecular mass markers are shown to the left of the gels, p*I* at the bottom of the 2D gels. In (D), the lanes represent: PK, proteinase K; BSA, bovine serum albumin; Dry, dry seeds; 21 d D, 21 d dark-stratification; 21 d L, 21 d light-stratification.

was stimulated slightly (1.5-fold) in the dark. Upon transferral to germination conditions for 24 h, peroxidase activity in the light-stratified seeds increased to the same level as that in the dark-stratified seeds (Fig. 3B).

Discussion

The role of α-amylase in stimulating germination

The original hypothesis that dark-stratified *Lolium rigidum* seeds may become more prepared for germination by mobilising storage reserves during stratification and providing energy for embryo expansion upon exposure to alternating light and temperature was not supported by the data, as there was no difference between dark- and light-stratified seeds in terms of carbohydrate and lipid concentration or activity of total amylase or ICL. α-Amylase activity, expressed constitutively in seeds selected for low dormancy (Goggin et al., 2010), was not induced during dark-stratification, demonstrating that it is not involved in dark-mediated dormancy release *per se*. However, α-amylase activity rapidly appeared (at least 48 h prior to radicle emergence) once the dark-stratified seeds had been transferred to alternating light and temperature, and thus this enzyme may be involved in stimulation of germination of *L. rigidum* seeds. The fact that exogenous GA_4 applied during light-stratification caused induction of α-amylase activity (Fig. 1D inset) suggests that the observed induction in dark-stratified seeds 24 h after transferral to germination conditions may be due to a progressive increase in GA sensitivity and/or concentration during dark-stratification, which becomes important once the seeds are transferred to light. However, the block to germination that exists in light-stratified seeds supplied with GA_4 (Goggin et al., 2009) and thus expressing α-amylase, which only ends upon transferral of the seeds to alternating light and temperature conditions, indicates that another (currently unknown) factor is also necessary for stimulation of germination.

There is some controversy over whether α-amylase is required for stimulation of seed germination or is merely important for providing soluble carbohydrates for post-germinative seedling growth (Asatsuma et al., 2005, and references therein). Although activity in non-dormant wheat seeds was only detected around 4 d after the start of imbibition (Perata et al., 1992), De Lespinay et al. (2010) found that α-amylase activity was present in dry seeds of *Poa pratensis*. Additionally, grass seeds in which α-amylase activity is inhibited are either much slower to germinate or incapable of germinating at all unless supplied with soluble sugars (e.g. Perata et al., 1992; Kato-Noguchi and Macías, 2008). Germination of transgenic rice lacking expression of α-amylase I-1 was also inhibited, and it was proposed that this scutellum-specific isoform is important for stimulation of germination, whilst the aleurone-localised isoforms of α-amylase are involved in post-germinative seedling growth (Asatsuma et al., 2005).

Light-stratification causes a net loss of storage proteins

Two-dimensional PAGE analysis revealed major differences in the effects of dark- and light-stratification on *L. rigidum* seed stor-

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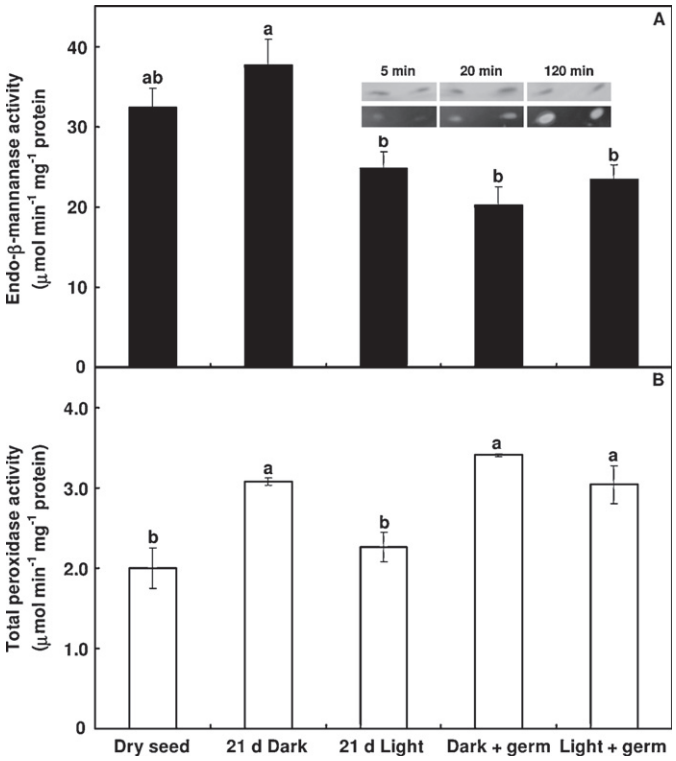

Fig. 3. Effects of stratification on seed endo-β-mannanase (A) and total peroxidase (B) activities. Activity was measured in dry seeds, immediately after 21 d stratification at 20 °C in the dark or light, or 24 h after transferral to germination conditions following stratification. Values are means ± SE ($n = 4$); the 5% LSD values are 2.8 for (A) and 0.5 for (B). The inset shows tissue-printing of cut seeds (imbibed for 24 h in the dark) that were incubated on galactomannan plates for 5, 20 or 120 min at 30 °C (bottom panels). The position of the seeds was recorded (top panels) before they were removed for colour development of the plates.

age proteins, with evidence of significant proteolysis occurring in the light. However, there was no obvious correlation between protein spot abundance and protease activity in a comparison of dark- vs. light-stratified seeds. It is possible that the intense, very high molecular mass protease bands that were unique to the light-stratified seeds were responsible for the greater extent of storage protein degradation. In walnut kernels, the proteases responsible for storage protein degradation during warm and cold stratification prior to germination were also of a very high molecular mass and more active at a neutral pH (Einali and Sadeghipour, 2007), similar to the high-intensity bands in the light-stratified *L. rigidum* seeds.

The difference in storage protein pattern between dark- and light-stratified seeds suggests that, rather than storage proteins being required as a source of amino acids and carbon during dark-mediated dormancy release, it is their continued presence as intact proteins that is important. The reason for this can currently only be speculated upon. The cupin superfamily to which the 7S vicilin-like storage proteins belong also contains non-storage proteins which are very similar in structure to the vicilins, can have the same patterns of accumulation and degradation as storage proteins, and are likely to have evolved from the same ancestral globulin (Wohlfarth et al., 1998). Examples include a 64 kDa sucrose binding protein of unknown function, and proteins with oxalate-degrading or superoxide dismutase activity (Dunwell et al., 2008). The two latter activities produce hydrogen peroxide and are associated with plant stress responses, so it is possible that the maintenance of storage (and storage-like) proteins in dark-stratified *L. rigidum* seeds could be related to defence against pathogens during stratification, or preparation for the most vulnerable stage of the seed life cycle, initiation of germination.

The role of cell wall hydrolysis in dormancy release

Endo-β-mannanase, active in extracts of dry *L. rigidum* seeds, maintained its activity during dark-stratification but was inhibited in the light, pointing to a possible role in dark-mediated dormancy release via weakening of the structures constraining the embryo. In contrast, β-mannosidase does not appear to mediate differential dormancy release in the dark and light. It has been proposed that cell wall polysaccharides in grass seeds are used as carbon resources during germination, as is the case in the date palm *Phoenix dactylifera* (reviewed in Buckeridge et al., 2000). In *L. rigidum* seeds, therefore, it is tempting to speculate that during dark-stratification, the higher activity of endo-β-mannanase is involved not only in weakening the structures surrounding the embryo, but also in providing substrates preparatory for rapid embryo expansion immediately upon perception of germination stimuli. This would leave the major carbohydrate and protein reserves in the endosperm, in which no dramatic changes were observed during dark-stratification, available for seedling growth after the completion of germination. Barrero et al. (2009) proposed the coleorhiza of barley seeds as the dormancy-regulating tissue, as it surrounds the embryonic roots and is the site of changes in abscisic acid sensitivity and cell wall-hydrolysing enzymes during dormancy release via after-ripening. Additionally, endo-β-mannanase activity was localised to the micropylar endosperm in *Datura ferox* seeds (Sánchez and de Miguel, 1997). In *L. rigidum*, however, activity appeared to be distributed over the whole seed. This may be due to the small size of the *L. rigidum* seed and the relatively poor resolving power of the gel diffusion method; alternatively, the universal localisation of endo-β-mannanase activity could be explained if it is indeed involved in substrate mobilisation for growth and expansion.

The activity of total peroxidase was slightly higher in dark-stratified seeds, suggesting that it may contribute to loosening of the endosperm and/or radicle cells walls and thus allow the radicle to emerge upon perception of germination stimuli. Although light-stratified seeds showed a similar increase in total peroxidase activity upon transferral to germination conditions, the fact that activity was stimulated prior to this in dark-stratified seeds supports its putative contribution specifically to dormancy release in non-germinating seeds.

Conclusions

Mobilisation of storage compounds in preparation for germination is not involved in dark-mediated dormancy release in *L. rigidum* seeds, but this study has shown that a number of processes, which individually may not seem to have a large impact on dormancy status, are simultaneously activated in the dark and/or inhibited in light. These include endo-β-mannanase and total peroxidase activities, which are slightly higher in the dark and may contribute to weakening of the structures surrounding the embryo prior to the perception of germination stimuli, maintenance of storage(-like) proteins in the dark through slightly lower induction of protease activity, and an apparent increase in sensitivity to GA (combined with the decrease in sensitivity to abscisic acid described in Goggin et al., 2009), which permits induction of potentially germination-stimulating α-amylase activity immediately upon sensing of light and alternating temperature.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jplph.2010.09.001.

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